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Phytochrome A as a functional marker of phyletic relationships in *Nicotiana* genus

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Abstract

Nicotiana is a small and well characterized genus of *Solanaceae* and in this study we have used polymorphisms in phytochrome A coding sequence (*phyA*) and promoter to asses the phylogenetic relationships among species representative of all the sections of the genus. Allopolyploid species kept the two copies of the gene derived from each of the progenitors as resulted from the analyses of the coding region and promoter. Moreover, both copies of *phyA* present in tetraploids are transcribed, indicating that are properly regulated and do not undergo silencing.

Additional key words: allopolyploidy, evolution, photoreceptors, polymorphism, promoter.

Introduction

Nicotiana is a small and well characterized genus of *Solanaceae*, native to Bolivia, which is widespread all over the New World including North America and Australia (Goodspeed 1954). According to Goodspeed, the evolutionary story of the subgenera of *Nicotiana* can be described at three fundamental levels: the genus was apparently evolved from an initial pregeneric reserve common to the genera *Cestrum* and *Petunia*. The *Nicotiana* species are the product of an evolutionary process which has conserved characters from both the progenitors. The genetic elements of the pre-*Nicotiana* were apparently channeled early into the groups of Cestroid and Petunioid, with the differentiation of the pre-subgeneric aggregates. In this phase of evolution the passage from the level of 6 chromosomes to that of 12 occurred. The aggregate with a predominantly Cestroid character gave origin to the subgenera pre-Rustica and pre-Tabacum while the pre-Petunia kept the characters of the Petunioid complex (for a schematic representation see Intrieri and Buiatti 2001). From the three ancestral subgeneric complexes the modern sections were derived. More recently, Chase and collaborators (2003) suggested a closer phylogenetic relation of the *Nicotiana* with the *Antocercids*.

Among living species, two further evolutionary levels are recognizable, the first with 12 pairs of chromosomes, the second amphidiploid with a genetic inheritance of 48 chromosomes, generated by duplication or by inter-specific hybridisation of the groups described. Finally in the genus there are also aneuploid species, in the section of the *Alatae* and *Sauveolentes*, derived from series with 12 or 24 pairs of chromosomes. In more recent evolutionary times, phenomena of introgression and inter-specific hybridization not followed by tetraploidization have also apparently contributed to the speciation. Therefore, speciation came about through amphidiploid and allopolyploid interspecific hybridization, more rarely through geographic isolation since most of the species are sympatric amongst themselves.

The systematic of the genus *Nicotiana* has been also studied, right up to the present day, through characterization of DNA multiplicity changes in the genus (Narayan 1987), analyses of the morphological and physiological behaviour of plantlets grown *in vitro* of several species (Bogani *et al.* 1985, 1997), studies of inter-specific sequence variations through the use of a series of molecular markers like chloroplast RFLP (Kung *et al.* 1982, Tassopulu and Kung 1984), repeated sequences (Hua *et al.* 1993, Borisjuk *et al.* 1994, Gregor *et al.* 2004, Skalicka *et al.* 2005), gene and neutral marker

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Abbreviations: AFLP - amplified fragment length polymorphism; ITS - internal transcribed spacer; PCR - polymerase chain reaction; Phy A - phytochrome A; RAPD - random amplified polymorphic DNA; RFLP - restriction fragment length polymorphism.
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polymorphism, such as RAPD and AFLP (Bogani *et al*. 1997, Yu and Lin 1997, Intrieri and Buiatti 2001, Ren and Timko 2001, Zhang *et al*. 2005), useful also in other species (Rout 2006, Mohapatra and Rout 2006), and finally to recent studies on chloroplastic *matK* (Aoki and Ito 2000), ITS sequences and chromosome architecture studies (Chase *et al*. 2003), and on allopolyploidy events during the evolution of the genus (Lim *et al*. 2004, Kovarik *et al*. 2004, Clarkson *et al*. 2005).

Since no functional gene marker has been analyzed in *Nicotiana,* the aim of the present work is to identify markers correlated with important traits for the ecology of the species and capable to show polymorphism in loci subject to natural selection (Kuittinen *et al.* 2002). Phytochromes are a family of biliprotein photoreceptors, included in a small family of genes that regulate a wide range of photomorphogenetic and adaptive responses through plant development, such as seed germination, flowering, senescence and de-etiolation, pigmentation, trophisms, shade avoidance, dormancy, and photosynthate partitioning (Kendrick and Kronenberg 1994). Therefore phytochromes are a good candidate to be considered as excellent functional markers for studying the evolution and differentiation of plants even if until now they have only been used at taxonomically higher levels (Alba *et al.* 2000, Matthews and Sharrock 1996, Matthews and Donoghue 1999, 2000) and for only a few species of the genus *Nicotiana* (Intrieri *et al*. 2004). In this work the authors present an analysis of phytochrome A polymorphism (*phyA*) in an wide number of species of the genus *Nicotiana*.

Materials and methods

Total DNA was isolated using the method reported in Intrieri *et al*. 2004, from fresh leaf tissue of *Nicotiana* spp. listed in Table 1. *Nicotiana* spp. were kindly provided by the Istituto Sperimentale per il Tabacco (Scafati, Salerno, Italy). PCR forward and reverse primer sequences used, designed on published *N. tabacum phyA* sequence (Adam *et al.* 1993, 1995), are *N. tabacum* chromofore binding site (fw: GTGACACTATGG TTCAGGAG; rv: GAGCTACTGGCATCAGCATA; annealing temperature, Ta 57 °C), *N. tabacum* 5'UTR (fw: GCTTGGTCTTGAAGATGACA; rv: GTGTAG AGTTGTCTTGCATG; Ta 55 °C), *N. tabacum* promoter (fw: TCATGCAAGACAACTCTACAC; rv: TCATGA Ga/gCTTTCCGa/gCATA; Ta 50 °C), *N. tabacum* T-Pe1-T-Pe3 region (fw: TTG GTTGTACAAAt/cGGCCAAA; rv: TGATt/gAGAAa/tACCCACTTGG; Ta 50 °C) *N. tabacum* T-Pe3 T-Gt2 region (fw: CCAAGT GGGTa/tTTCTa/cATCA; rv: GAAGGCTTCTTATGT CAAc/gA; Ta 50 °C).

Gene amplifications, PCR product direct restriction and fragments visualisation were performed as previous described (Intrieri *et al*. 2004). From the data obtained with the restriction enzymes, similarity matrices were constructed, based on the reading of the restriction profiles and on the preliminary construction of matrices of presence, 1, and absence, 0, of the bands visualized on gel. The data were processed by means of *NTSYS-pc2.02i* (*Exeter Software*, New York, USA) for matrix computation and to obtain phylogenetic grouping.

Results and discussion

In all the species examined the expected fragments of the *phyA* gene were amplified. The PCR amplifications of all the species underwent restriction reactions with enzymes chosen on the basis of the sequence of *N. tabacum*. The enzymes EcoRV, EcoRII, NsiI, AvaII and MboI did not display restriction site polymorphism. Polymorphic restriction profiles were obtained with the enzymes HindIII, TaqI, AluI, ScrFI, Tru91, BanII and Ddel. The enzyme Tru91 gave rise to polymorphic bands in only two species, and these were related to each other: *N. glauca* and *N. cordifolia*, indicating site loss, with respect to their last progenitor, probably occurring before or during the differentiation of the two species from the latter. BanII profiles resulted polymorphic in only *N. umbratica*, indicating a recent mutation. The enzyme ScrFI is polymorphic only in one of the 3 subgenera of the genus, the subgenus Petunioides. The species *N. trigonophylla* has a pattern of 2 bands ascribable to site loss compared to the sequence of *N. tabacum* which has a pattern of 3 bands. *N. trigonophylla* is a species found in the central nucleus of the genus, probably one of the most ancestral species. According to the classic systematics of *Nicotiana*, this species introgressed into the *Repandae*, and into other sections of the Petunioides. The *Repandae*, all tetraploids, confirm the introgression, presenting both the pattern of bands originating from *N. trigonophylla* and the more common one of the genus, represented by *N. tabacum*. The typical pattern of *N. trigonophylla* is observed in *N. nudicaulis*, *N. undulata* and *N. exigua*. A unique and characteristic pattern, on the other hand, is seen in *N. clevelandi*. The restriction product of HindIII has highlighted the appearance of a site in the evolutionarily close sections of *Noctiflorae*, *Acuminatae* and *Bigelovianae*. The mutation could have originated in the *Noctiflorae* or in the *Acuminatae*, in so far as the *Bigelovianae* are tetraploid species, according to Goodspeed (1954), originating from the *Acuminatae*.

The restriction profiles obtained with TaqI indicate the presence of two of the most common forms of the analyzed fragment, one with the three bands of 371, 220

and 24 bp, and a second one with bands of 290, 220 and 115 bp. The two forms are present in *N. tabacum*, being derived from the species *N. tomentosiformis* and *N. sylvestris* which contain the first and second forms, respectively. Moreover, there are forms particularly characteristic of only a few species, like that of *N. rustica* and *N. undulata* and the typical form of *N. knightiana* (500 and 115 bp) and of some *Suaveolentes*. *N. rustica* is an allopolyploid that Goodspeed (1954) suggested was derived from *N. undulata*, a hypothesis confirmed by Lim *et al*. (2004). The enzyme AluI furnishes a further example of the variability present in the genus *Nicotiana*. In this case the restriction profiles can be traced back to 4 forms of the gene present in combination in the allopolyploids. For AluI, as for the enzyme TaqI, the greatest variation is concentrated in the subgenera Rustica and Tabacum and in the subgenera Petuniodes in the section of the *Suaveolentes*.

From the similarity matrices, with the *Ntsys* software, a UPGMA tree was obtained, based on the data from the polymorph enzymes. The similarity matrices were obtained using the simple matching formula. The tree of consent obtained with the algorithm UPGMA (Fig. 1) allows observations to be made and the phenogram is divided into two main clusters, one containing the species belonging to the sections of the *Noctiflorae*, *Acuminatae* and *Bigelovianae*, and the other containing the remaining species. The latter cluster subdivides in turn into two other main clusters, one groups together the species of the subgenus Tabacum and the other the remaining Petunioides. The distribution of the species in the tree emphasizes the phenomena of introgression already suggested by Goodspeed (1954) himself. The *Repandae* are united in one sole cluster, together with the *Nudicaules*. A fact worth noting that emerges from looking at the tree is the dispersion in diverse clusters of the subgenus Rustica. A possible explanation is coherent with Goodspeed's assertion, that this is the case of an ancient subgenus, while another one could be found in the ability of this species to hybridize itself with members of other subgenera. Thus, the species *N. glauca*, closely related to *N. cordifolia*, clusters together with the *Noctiflorae,* although, it is not related with the latter it could be repeatedly introgressed, according to Goodspeed (1954). This is in agreement with the distribution of the species in the phenogram constructed with the *matK* (Aoki and Ito 2000) and ITS (Chase *et al*. 2003) marker data. Similarly, the species *N. benavidesi* is close to the species of the subgenus Tabacum with which it hybridizes in nature. In fact the species *N. glutinosa* and *N. benavidesi*, while classified in different sections, hybridized repeatedly during their evolution to the point where they had characteristics in common (leaf and trichrome shape) (Goodspeed 1954). In our tree the two species are united in a cluster that also includes members of species of the subgenus Petunioides. It is worth noting the fragmentation of the *Suaveolentes* section which groups together all the Australian species. In this case the interpretation indicates that the *Suaveolentes* originated by allopolyploidy from ancestors close to the present *Alatae* and *Acuminatae* or *Noctiflorae*; consequently the origin of the section is not monophyletic but polyphyletic (Goodspeed 1954). The polyphyletic origin of the section is clearly seen in the tree that the present Authors constructed of the polymorphism of *phyA* but not in that constructed with the *matK* (Aoki and Ito 2000) and ITS (Chase *et al*. 2003) data, probably because the latter data highlight only the polymorphism of one of the parents. The *matK* polymorphisms are obtained from the chloroplasts genome analysis (maternal), while those of ITS could be affected by the silencing of the genomic region, where they are found, often involving the rDNA of paternal origin, both hypotheses already put forward by Chase and his collaborators (2003). The data obtained from the two orthologous phytochromes, which are both conserved in the allopolyploids of *Nicotiana*, are able to highlight the polyphyly. The position of *N. rustica* close to representatives of the *Petunioides* is explained, as

Table 1. *Nicotiana* species analyzed.

Subgenus	Section	Species	2n	Author	No.
Rustica	*Paniculatae*	*glauca*	24	Graham	1
		knightiana	24	Goodspeed	2
		solanifolia	24	Walpers	3
		benavidesi	24	Goodspeed	4
		cordifolia	24	Philippi	5
		raimondi	24	Macbride	6
	Rusticae	*rustica*	48	Linnaeus	7
Tabacum	*Tomentosae*	*tomentosiformis*	24	Goodspeed	8
		othophora	24	Grisebach	9
		setchelli	24	Goodspeed	10
		glutinosa	24	Linnaeus	11
	Genuinae	*tabacum*	48	Linnaeus	12
Petunioides	*Undulatae*	*undulata*	24	Ruiz and Pavon	13
		arentsi	48	Goodspeed	14
	Trigonophyllae	*trigonophylla*	24	Donal	15
	Alatae	*sylvestris*	24	Spegazzini	16
		langsdorffi	18	Weinmann	17
		alata	18	Link and Otto	18
		forgetiana	18	Hamsley	19
		longiflora	20	Cavanilles	20
		plumbaginifolia	20	Viviani	21
		sanderae	18		22
	Repandae	*repanda*	48	Willdenow	23
		stocktoni	48	Brandegee	24
		nesophila	48	Johnson	25
	Noctiflorae	*noctiflora*	24	Hooker	26
		petunioides	24	Millan	27
	Acuminatae	*acuminata*	24	Hooker	28
		pauciflora	24	Remy	29
		miersi	24	Remy	30
	Bigelovianae	*bigelovi*	48	Watson	31
		clevelandi	48	Gray	32
	Nudicaules	*nudicaulis*	48	Watson	33
	Suaveolentes	*umbratica*	46	Burbdige	34
		debneyi	48	Domin	35
		gossei	36	Domin	36
		suaveolens	32	Wheeler	37
		exigua	32	Wheeler	38

already mentioned, by assuming that it is a case of a allopolyploid originating from *N. undulata*. A similar phenomenon, although less accentuated, is found in the tree constructed with the RAPD data (Bogani *et al*. 1997), while in the tree constructed with the chloroplastic sequences of *matK, N. rustica* is positioned near to the maternal progenitor *N. paniculata*. Finally, in the tree the *Repandae* are close to *N. nudicaulis* as with the tree constructed with the ITS data (Chase *et al*. 2003).

The upstream sequence of the *phyA* gene in *N. tabacum* consists of a portion immediately upstream of the gene until 1000 bp from the ATG, namely the 5'UTR, and a subsequent portion until 1800 bp further upstream of the gene, containing the regulatory boxes T-PE-1 and T-PE-2 and the T-GTE2 (Fig. 2), responsible for the regulation through specific transcription factors (Adam *et al.* 1995). To investigate whether the presence of the two gene copies of the progenitors, are similarly preserved in the promoters, the zone upstream of the gene was subdivided into two fragments, adjoining each other, and amplified by PCR. The primers constructed on the *N. tabacum phyA* promoters were not able to produce amplification products in some species examined. This is coherent with an expected greater variability in the non coding sequences compared to that found in the coding ones. The variability in length of this region was shown to be rather high (~800/~1000 bp). However, it is possible to group together bands of similar size, that in most of the cases are coherent with the phylogenesis of the species. Also in the case of the promoter it is possible to find in the allopolyploid species the two copies of the promoters of the gene derived from the progenitors. If the case of *N. tabacum* is emblematic than this species shows two bands identical in dimensions to those of the two progenitors, therefore the allopolyploid sections, the *Repandae*, the *Bigelovianae*, the *Nudicaules* and the *Suaveolentes*, also show a couple of bands (Fig. 2), each band traceable to that of the putative progenitors suggested by Goodspeed (1954). These cases concern older tetraploidization events, therefore it is not always possible to go back exactly to the progenitor, which in the course of tens of millions of years could, in its turn, have changed: this is the case of the *Suaveolentes* that display a heterogeneous pattern, although similar to the bands of *Alatae*, *Noctiflorae* and *Acuminate*. As already stated, the *Suaveolentes* have a polyphyletic origin anyway. The 5'UTR region is amplificable in almost all the species examined, apart from *N. rustica* and *N. undulata*, (the first allopolyploid of *N. paniculata* and *N. undulata* itself; even *N. paniculata*, not included at first in the analyses but examined later, did not provide any sign of amplification). In this case also, the allopolyploids showed double bands, even though not always traceable to the putative progentiors. The zone containing the inducible T-PE1 T-PE2 and T-GPE2 boxes confirms the data obtained from the largest fragment

The analysis of the gene fragment in the region coding for the chromophore binding site shows that the

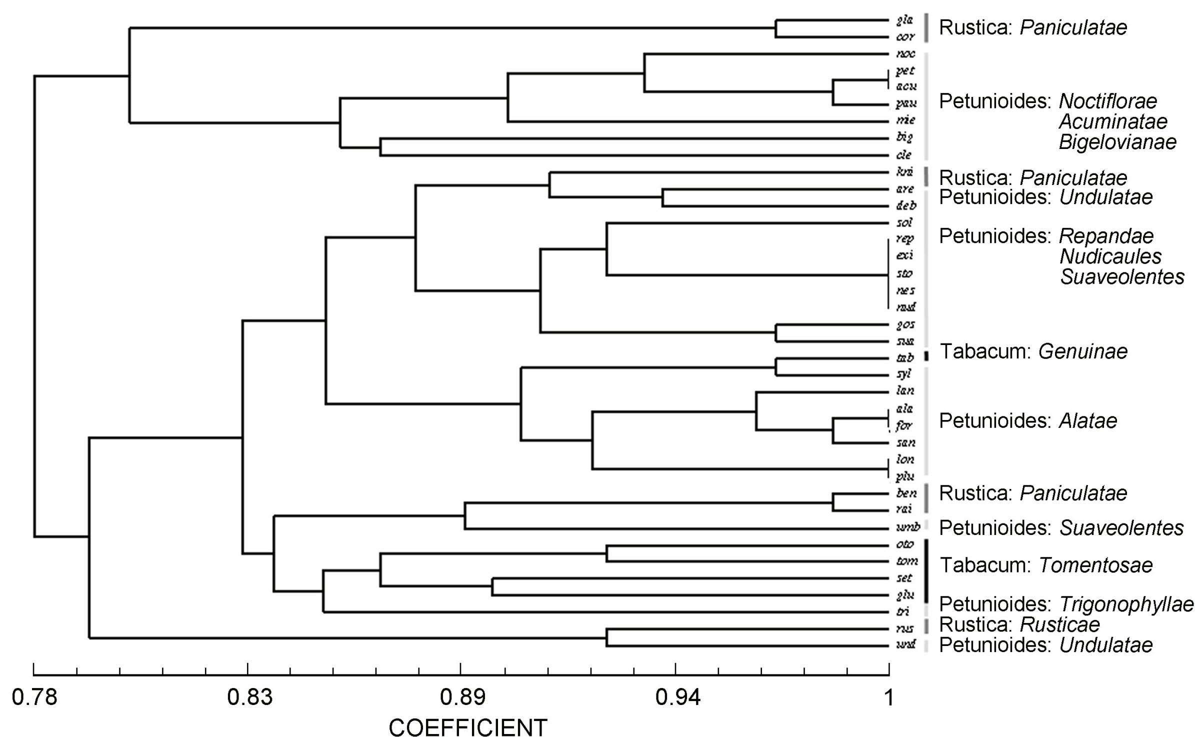

Fig. 1. Dendrogram of *Nicotiana* species obtained from *phyA* restriction data with the algorithm UPGMA. On the side of the abbreviation of the species name are reported the subgenus and sections as classified by Goodspeed (1954).

PROMOTER
CODING SEQUENCE
putative transcription start sites
T-PE1 T-PE3 T-GT2 ATG chromophore binding site
1 2 3 4 5

1

bp	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
900																																			+			
881		+																+					+	+	+							+		+	+	+	+	
824								+				+			+		+					+									+	+	+					
800			+						+	+	+	+				+							+	+	+								+	+		+		
780				+		+	+																					+	+	+	+						+	

2

bp	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
908								+	+			+			+		+	+	+			+									+					+	+	
868		+								+		+				+																	+					+
800	+	+	+	+	+	+								+									+	+	+	+	+	+	+	+	+	+			+	+	+	

3

bp	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
193		+		+			+	+	+		+	+	+	+				+	+			+	+	+	+													+
180	+		+			+	+					+	+	+	+	+	+												+	+	+	+	+	+				
170					+					+													+	+	+			+	+	+	+	+			+	+	+	
160																										+	+											+

4

bp	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
460							+						+																					+				
394				+		+	+	+	+	+	+	+	+				+	+	+			+																
330					+							+		+	+	+				+	+	+	+	+	+	+	+				+	+	+		+			+
310	+	+	+											+									+	+	+				+	+	+					+	+	+
295																																						

Fig. 2. Schematic representation of *phyA* gene structure. The *bidirectional arrows* indicate the regions analysed among the different species: 1 - amplicon of 5'UTR; 2, 3, 4 - amplicons of promoter regions; 5 - amplicon of the codifying chromophore region. In bold are indicated the sizes expected from the *N. tabacum* sequence (number 12). The number of each species is the same of that reported in Table 1.

species originating through allopolyploidy maintained the copies of the gene derived from each of the progenitors. *phyA* has a crucial role in the monitoring of environmental light, in the synchronization of circadian rhythms and in floral induction and in the reply strategies that the plant activates in order to adapt its development to the environment. In this work we show that the two copies of *N. tabacum phyA* gene, previously showed by Adam *et al*. (1995) to be both transcribed, are derived from the ancestral progenitors. This result seems to be in contrast with many studies reviewed in literature (Adams and Wendel 2005, Comai 2005), where it is proposed that most duplicated genes are silenced after polyploidization. Our results reported here allow one to envisage a different pattern of evolution of genes that from the tetraploidization event onwards functioned in regulated and coordinated way.

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